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Chemotherapy, Neurotoxicity, and Cognitive Decline: Developing
a Mouse Model and Potential Interventions

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14. ABSTRACT Adjuvant chemotherapy often causes cognitive decline in breast cancer survivors. Although the cognitive deficits are often temporary, it appears that for at least a subset of survivors, the deficits last for years and can have a deleterious impact on survivor quality of life. Recent evidence shows that chemotherapy agents can have long-lasting neurotoxic effects: increase in cell death and decrease in cell division/proliferation in the SVG, the DG, and the CC, as well as delayed myelin degeneration. Which chemotherapy agents or combinations of agents cause CNS damage remains unclear. Our study was designed to determine 1) if doxorubicin or cyclophosphamide cause a decrease in neurogenesis and/or myelin damage and 2) if neurogenesis and/or myelin damage caused by 5-Fluorouracil can be prevented by pre and co-treatment with antidepressants or antioxidants. The results from our auditory brainstem response experiments suggest that 5-Fluorouracil and cyclophosphamide cause a transient speed of processing deficit, while doxorubicin does not. In addition, our results suggest that co-treatment with antioxidants does not prevent the transient speed of processing deficit. Our immunocytochemistry experiments are ongoing and will help us determine if doxorubicin or cyclophosphamide cause a long lasting decline in neurogenesis, as does 5-Fluorouracil, and if antidepressants or antioxidants can prevent such damage.					
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Introduction

Adjuvant chemotherapy often causes cognitive decline in breast cancer survivors. Although the cognitive deficits are often temporary, it appears that for at least a subset of survivors, the deficits last for years and can have a deleterious impact on survivor quality of life. Recent evidence shows that chemotherapy agents can have long-lasting neurotoxic effects: increase in cell death and decrease in cell division/proliferation in the SVG, the DG, and the CC, as well as delayed myelin degeneration. Thus, at least one underlying mechanism of chemotherapy-induced cognitive dysfunction may be a decline in neuro and glialgenesis, and/or delayed myelin degeneration. With this in mind, a critical question becomes *what can prevent/reduce that negative mechanism **before** it occurs rather than waiting to try to alleviate the problem after the damage has occurred?* Given that most antidepressants cause an increase in neuro/glialgenesis it seems plausible that antidepressants might reduce some of the neurotoxic effects listed above, and thereby reduce cognitive decline. In addition, given that chemotherapy agents have, at least to some extent, a free radical mechanism, it seems plausible that antioxidants might reduce the neurotoxic effects of chemotherapy. Melatonin appears to protect against demyelination in general and to stimulate neurogenesis and is thus a good candidate antioxidant. Our research project takes advantage of the recent development of the mouse models of chemotherapy induced neurotoxicity to explore the possibility that antioxidants and antidepressants might prevent or decrease the severity of chemotherapy-induced long-lasting neurotoxicity.

Body

Aim 1: Can antioxidant or antidepressant treatment prevent or decrease the neurotoxicity caused by Fluorouracil (5-FU) treatment in mice.

1a. Measurement of **cell death** in the lateral subventricular zone (SVZ), the corpus callosum (CC), and the dentate gyrus (DG) at 1 day, 14 days, 56 days, and 6 months after 5-fluorouracil (5-FU) treatment using terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) as a function of pre & co-treatment with 1) N-acetyl cysteine (NAC) 2) Melatonin & 3) Fluoxetine.

Saline Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

5-FU Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

5-FU + NAC Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

5-FU + Melatonin Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

5-FU + Fluoxetine Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

1b. Measurement of **cell proliferation** in the SVZ, the CC, and the DG at day 1, 14 days, 56 days, and 6 months after 5-FU treatment using Ki-67 as a function of pre & co-treatment with 1) NAC 2) Melatonin & 3) Fluoxetine.

1c. Measurement of **white matter density** in the CC and white matter tracts of the striatum at 1 day, 14 days, 56 days, and 6 months after 5-FU treatment using optical density of anti-myelin basic protein (Anti-MBP) as a function of pre & co-treatment with 1) NAC 2) Melatonin & 3) Fluoxetine.

1d. Measurement of **transcriptional regulation** in oligodendrocytes at 1 day, 14 days, 56 days, and 6 months after 5-FU treatment assessed with labeling for both Olig2 and CC-1 as a function of pre & co-treatment with 1) NAC 2) Melatonin & 3) Fluoxetine.

1e. Measurement of **myelin function** prior to treatment, and at 1 day, 14 days, 56 days, and 6 months after 5-FU treatment as assessed by auditory brainstem response (ABR) as a function of pre & co-treatment with 1) NAC 2) Melatonin & 3) Fluoxetine.

Saline Group: (n=8 C57BL/6J mice)

5-FU Group: (n=8 C57BL/6J mice)

5-FU + NAC Group: (n=8 C57BL/6J mice)

5-FU + Melatonin Group: (n=8 C57BL/6J mice)

5-FU + Fluoxetine Group: (n=8 C57BL/6J mice)

We have completed the majority of data collection and data analysis for the auditory brainstem response (ABR) experiment in Aim 1 (i.e., Aim 1-1e.). The ABR is a robust response making it a useful technique in both clinical and research settings for studying disorders of the CNS, including myelin damage. Previously it was shown that 5-FU caused both myelin damage and a corresponding increase in ABR inter-peak latencies in mice. **Figure 1** shows a representative ABR from one of our mice. There are 5 main peaks of interest in the ABR indicated by the green, red, blue, purple, and yellow lines. The main indication of myelin damage is an increase in the distance between the peaks, although we have also analyzed other measures such as peak width and amplitude. **Figure 2** summarizes the various measures we use, although it should be noted that it shows the analysis for just the first 2 peaks.

Fig. 1

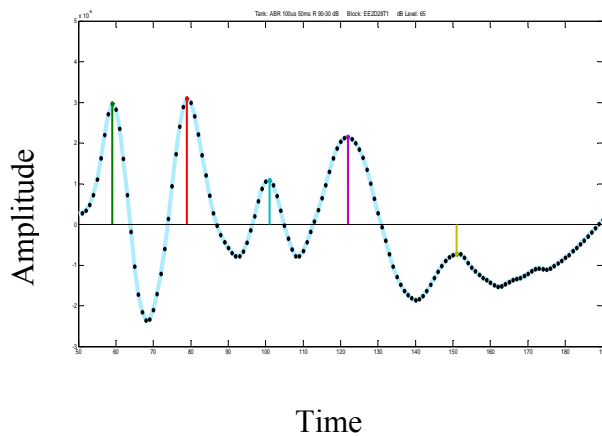
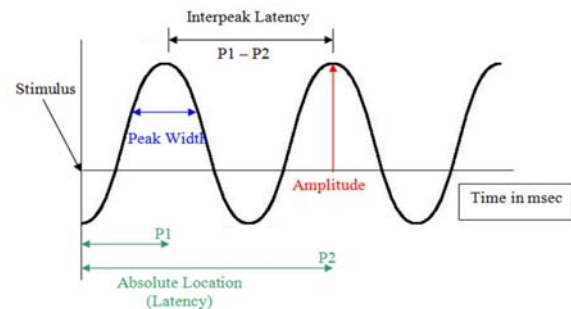


Fig. 2



We treated mice ($n = 8$ per group) with either 5-Fluorouracil (70 mg/kg) + Saline (0.9%), 5-Fluorouracil (70 mg/kg) + Melatonin (25mg/kg), 5-Fluorouracil (70 mg/kg) + N-acetyl-cysteine (NAC) (400mg/kg), or Saline (0.9%) + Saline (0.9%) and recorded ABRs.

Previously we had not completed the 6 month time point and noted in the previous report that because of unexpected animal death our sample size was lower than proposed. We have now collected the 6 month time-point from our original group of animals and have collected additional data from a second set of animals at the day 1 and day 14 time points. We will collect day 56 and 6 months for the second set of animals when they reach those time points.

As shown in **Figure 3**, the chemotherapy treated groups lost significantly more weight than did the control animals indicating therapeutic (i.e., toxic) effect (as did the loss of some animals).

Fig. 3

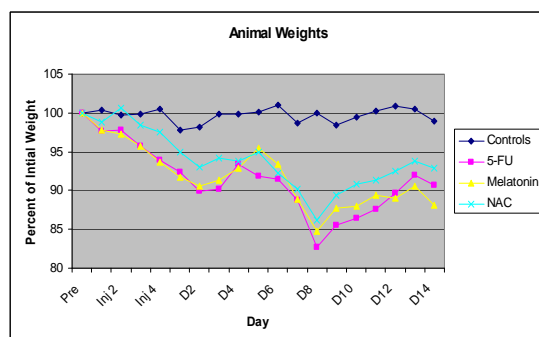


Figure 4 shows change in interpeak latency (peak 4 – peak 1) on day 1, 14, 56 and at 6 months. The change is relative to baseline data collected 2 days prior to treatment. A positive change indicates a slowing down of the ABR and suggests myelin damage. A negative change indicates a speeding up of the ABR and suggests enhancement of transmission speed. In general, the more peaks included in the interpeak analysis the more sensitive it should be to myelin damage, however, peak 5 is less stable than peak 4. Thus, in general, we have included our results for peak 4 – peak 1 rather than peak 5 – peak 1. As seen in **Figure 4**, 5-FU caused impaired auditory processing (5-FU treated animals were significantly slower than the control animals) at day 1, 14 and 56. Unfortunately, with the possible exception of the Melatonin group on day 14, the antioxidant treatments were not preventative. At 6 months there was a trend for the 5-FU + NAC group to be more impaired than the other groups, however, the 5-FU and 5-FU + Mel groups appeared to perform as well as the controls suggesting that 5-FU does not cause a permanent processing impairment. Consistent with the overall pattern seen at 6 months in the p4-p1 data we found that the 5-FU + NAC group was impaired relative to the control group and that neither the 5-FU nor the 5-FU + Mel groups were impaired relative to the controls. Thus 5-FU appears to cause a long lasting (at least 56 days) but not permanent speed of processing deficit. In addition, neither Mel nor NAC seems to prevent the impairment. In fact, NAC may prolong the impairment. We have not included the data from day 1 or 14 of the second group in the graphs shown in **Figure 4**. The data from day 1 and day 14 from the second group appears to be consistent with the first 2 graphs in **Figure 4**. However, that preliminary analysis was not run by our blind coder. Our blind coder is currently analyzing the data.

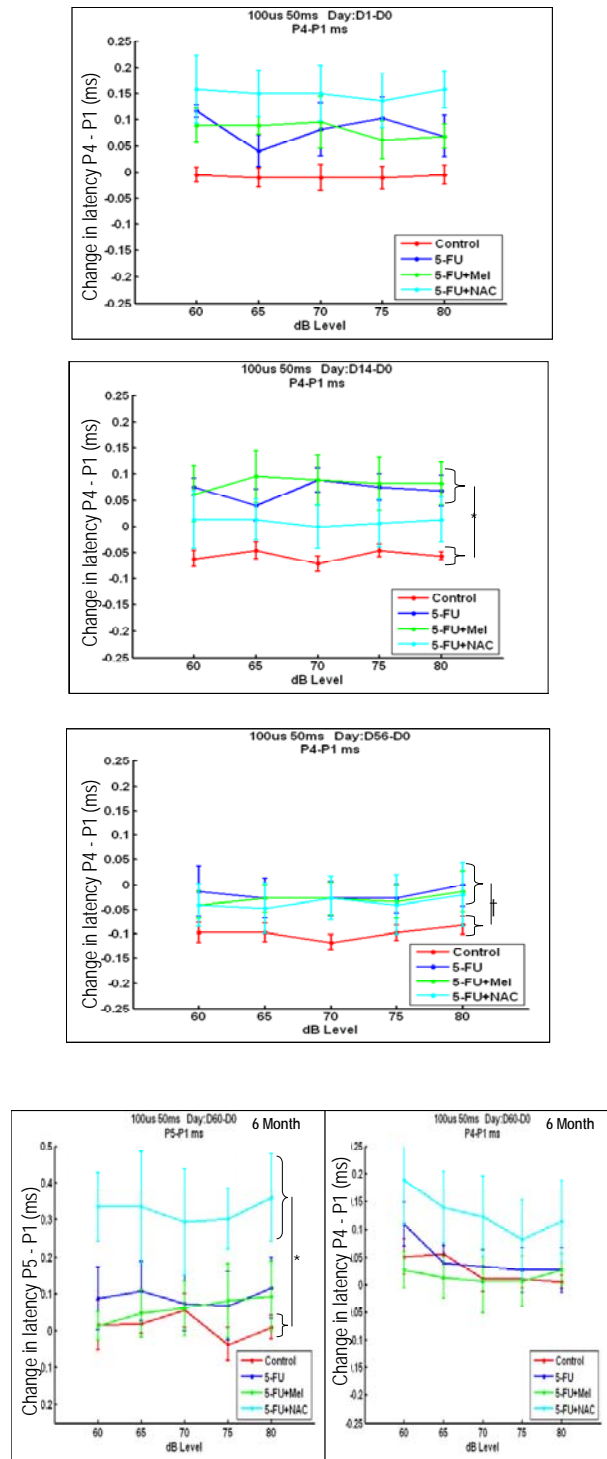


Figure 4: Change in P4 – P1 interpeak latency compared to day 0 for days 1, 14, 56, and 6 months: top to bottom figures. Positive scores indicate an increase in interpeak latency and negative scores indicate a decrease in latency. Error bars represent ± 1 standard error of the mean.

Day 1 5-FU, 5-FU+Mel, & 5-FU+NAC > Control all $ps < .05$;

Day 14 5-FU, 5-FU+Mel > Control all $ps < .003$

Day 56 5-FU, 5-FU+Mel, & 5-FU+NAC as a group > Control $p < .05$

6 Months No Significant differences for p4 - p1

6 Months 5-FU+NAC > Control $p < .02$ for p5 - p1

Previously we had perfused, frozen, and begun slicing brains for half the animals in our immunohistochemistry experiments (Aim 1 a-d) for the 1, 14, and 56 day time-points. In addition, half the animals for the 6 month time point had been treated and we were waiting for them to reach the 6 month time-point. We have now perfused, frozen and sliced the brains for all the animals for the 1 day, 14 day, and 56 day time points and for half the animals in the 6 month time-point. The other half of the animals for the 6 month time-point have been treated, but we are still awaiting the 6 month time point for perfusion. We have begun staining for Aims 1 a-d but have only about 1 animal per groups at this point. We anticipate that the staining will be complete for all groups within 5 months with the exception of the second group at the 6 month time-point.

Aim 2: Do doxorubicin & cyclophosphamide cause neurotoxicities similar to that of 5-FU in mice?

2a. Measurement of **cell death** in the SVZ, the CC, and the DG at 1 day, 14 days, 56 days, and 6 months after doxorubicin or cyclophosphamide treatment using TUNEL.

Saline Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

Doxorubicin Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

Cyclophosphamide Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

2b. Measurement of **cell proliferation** in the SVZ, the CC, and the DG at 1 day, 14 days, 56 days, and 6 months after doxorubicin or cyclophosphamide treatment using Ki-67.

2c. Measurement of **white matter density** in the CC and white matter tracts of the striatum at 1 day, 14 days, 56 days, and 6 months after doxorubicin or cyclophosphamide treatment using optical density of Anti-MBP.

2d. Measurement of **transcriptional regulation** in oligodendrocytes at 1 day, 14 days, 56 days, and 6 months after doxorubicin or cyclophosphamide treatment assessed with labeling for both Olig2 and CC-1.

2e. Measurement of **myelin function** prior to treatment, and at 1 day, 14 days, 56 days, and 6 months after doxorubicin or cyclophosphamide treatment as assessed by ABR.

Saline Group: (n=8 C57BL/6J mice)

Doxorubicin Group: (n=8 C57BL/6J mice)

Cyclophosphamide Group: (n=8 C57BL/6J mice)

We have completed data collection and the majority of data analysis for the auditory brainstem response (ABR) experiment in Aim 2 (i.e., Aim 2-2e.). The ABR is a robust response making it a useful technique in both clinical and research settings for studying disorders of the CNS, including myelin damage. Previously it was shown that 5-Fu caused both myelin damage and a corresponding increase in ABR inter-peak latencies in mice. **Figure 5** shows a representative ABR from one of our mice. There are 5 main peaks of interest in the ABR indicated by the

Fig. 5

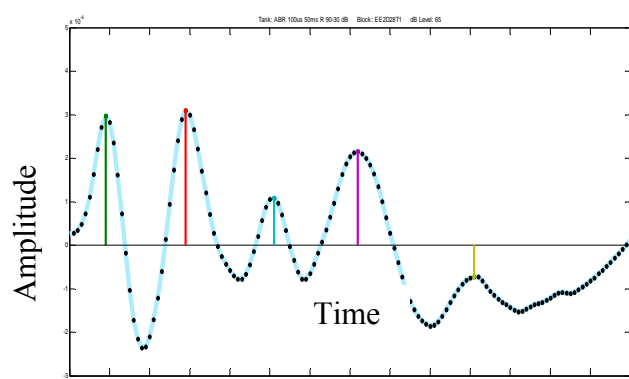


Fig. 6

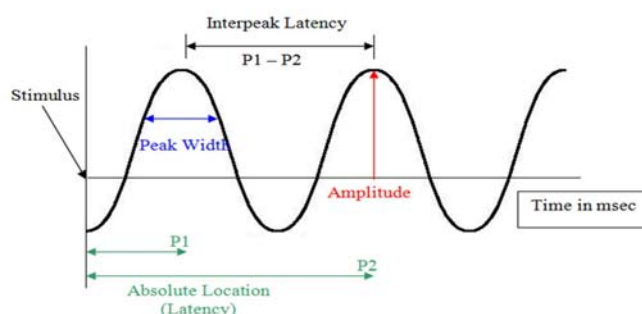
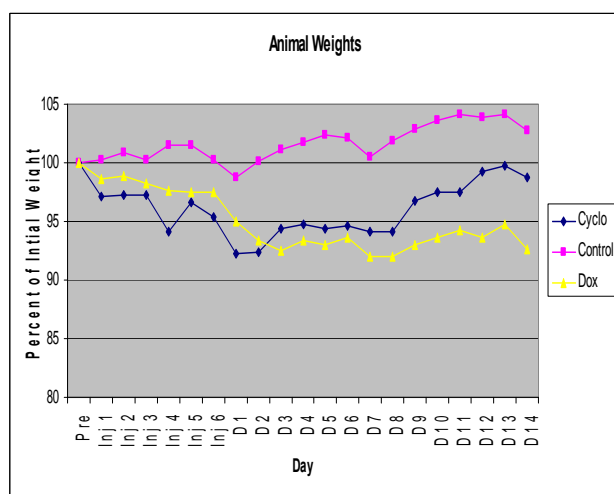


Fig. 7



green, red, blue, purple, and yellow lines. The main indication of myelin damage is an increase in the distance between the peaks, although we have also analyzed other measures such as peak width and amplitude. **Figure 6** summarizes the various measures we use, although it should be noted that it shows the analysis for just the first 2 peaks. We treated mice with either cyclophosphamide (120 mg/kg per i.p. injection; n=8), doxorubicin (5 mg/kg per i.p. injection; n=8) or saline (0.9%; i.p.; n=8) and recorded ABRs. Previously we had completed collecting and analyzing days 1, 14, and 56. We have now completed our last time-point (6 months) as well. As shown in **Figure 7** the chemotherapy treated group lost significantly more weight than did the control animals indicating therapeutic (i.e., toxic) effect. In addition, 5 of the 8 cyclophosphamide treated animals had gray hair by 4 months whereas none of the control animals did. Finally, 3 doxorubicin treated animals showed significant weight loss during months 4 and 5 and we had to place them on a liquid diet. Despite the significant toxic effects of the chemotherapy, we found no evidence of differences in the ABRs at the 1 day, 14 day, 56 day or 6 month time-points between the control animals and the doxorubicin or cyclophosphamide treated animals. **Figure 8** shows change in interpeak latency (peak 4 – peak 1) on day 1, 14, and 56 and at 6 months. The change is relative to baseline data collected 2 days prior to treatment. A positive change indicates a slowing down of the ABR and suggests myelin damage. A negative change indicates a speeding up of the ABR and suggests enhancement of transmission speed. In general, the more peaks included in the interpeak analysis the more sensitive it should be to myelin damage, however, peak 5 is less stable than peak

4. Thus, in general, we have included our results for peak 4 – peak 1 rather than peak 5 – peak 1. As seen in **Figure 8**, there were no significant differences between the control group and either

chemotherapy group. There was, however, a significant difference between the doxorubicin group and the cyclophosphamide group at day 1. Our dosing of Doxorubicin and Cyclophosphamide was based on previous reports in the literature and our own pilot dosing studies with a goal of creating about equal weight loss in both groups. Although the weight loss was about the same between the Doxorubicin and Cyclophosphamide groups, our pilot dosing studies indicated that the Doxorubicin dose was close to the upper-limit tolerated by the mice (as was our 5-FU dose), however, the Cyclophosphamide dose was not. As a result we ran a high dose Cyclophosphamide group (close to double our original dose and close to the upper-limit tolerated by the mice). **Figure 9** shows our results from the High Dose Cyclophosphamide group. We have not completed our analysis of day 56 and are awaiting the 6 month time-point for our last recoding, however, Figure 9 shows that there is at least a transient speed of processing deficit in the High Dose Cyclo group.

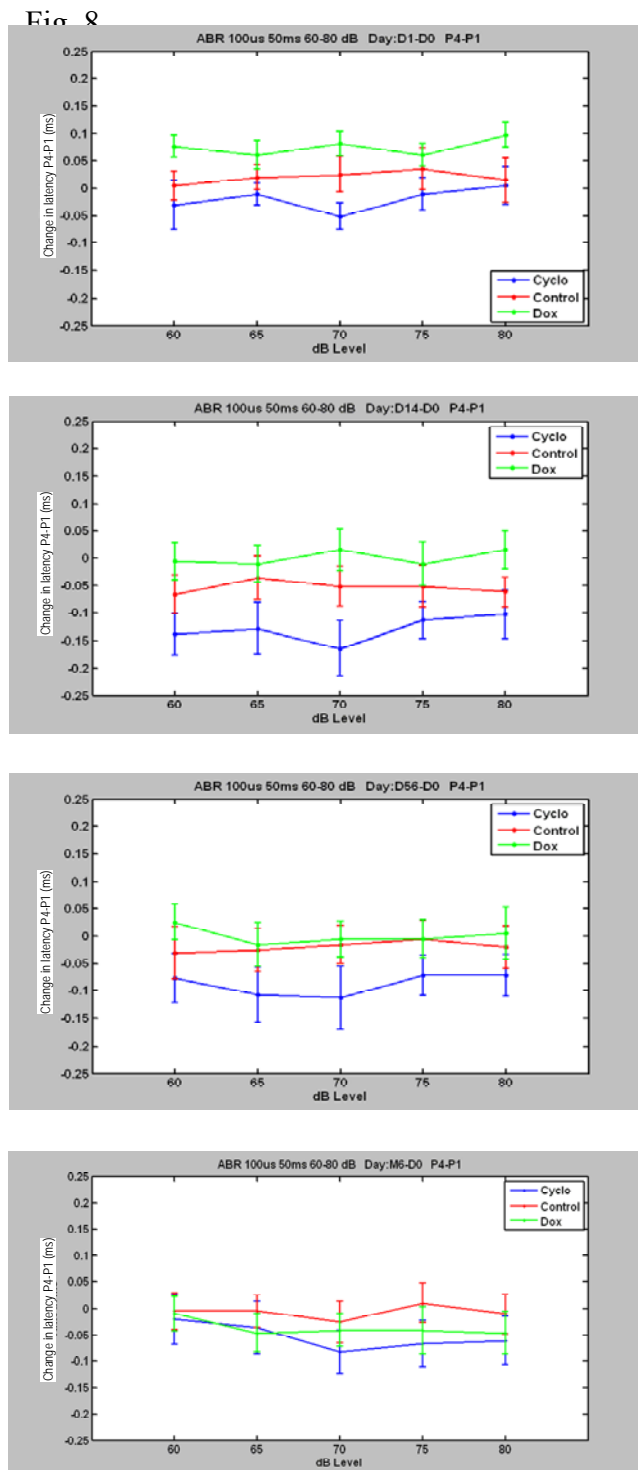


Figure 8. Change in P4 – P1 interpeak latency compared to day 0 for days 1, 14, 56, and 6 months top to bottom. Positive scores indicate an increase in interpeak latency and negative scores indicate a decrease in latency. Error bars represent ± 1 standard error of the mean. Doxorubicin (Dox) & Cyclophosphamide (Cyclo)
 Day 1 Dox > Cyclo $p < .02$;
 Day 14 Dox > Cyclo $p < .02$;
 Day 56 No significant differences
 6 Months No significant differences

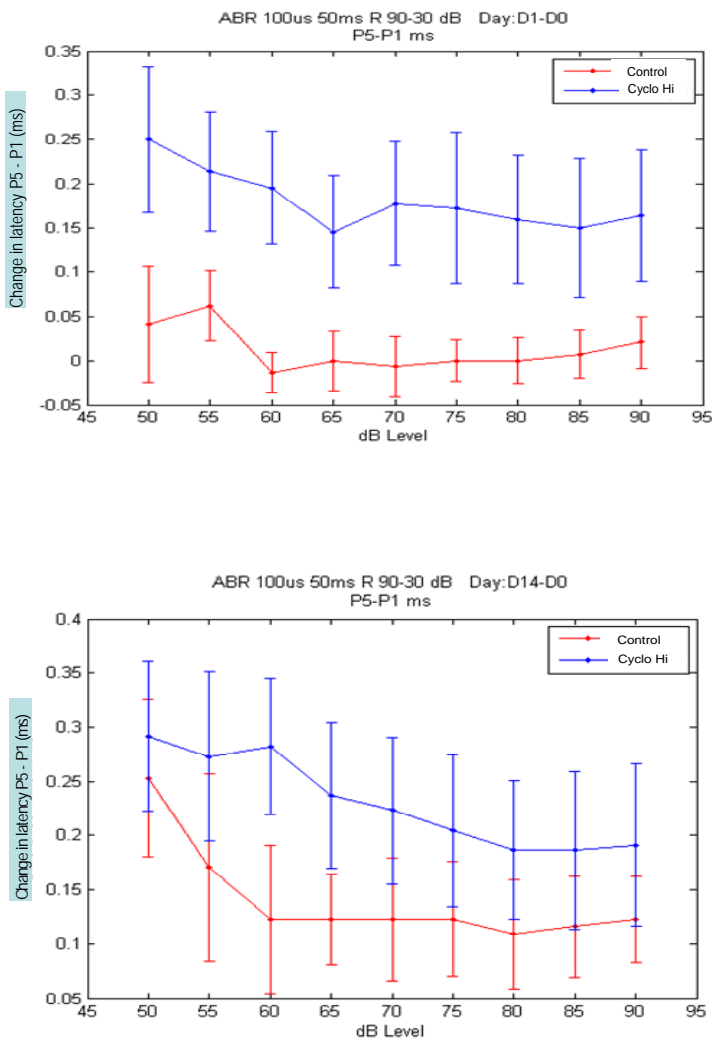


Figure 9. Change in P5 – P1 interpeak latency compared to day 0 for days 1 and 14 top to bottom . Positive scores indicate an increase in interpeak latency and negative scores indicate a decrease in latency. Error bars represent ± 1 standard error of the mean. Hi Dose Cyclophosphamide (Hi Cyclo)
 Day 1 Hi Cyclo > Control $p < .01$;
 Day 14 A trend for the Hi Cyclo > Controls

Previously we had perfused, frozen, and begun slicing brains for half the animals in our immunohistochemistry experiments (Aim 2 a-d). We have now perfused, frozen and sliced the brains for all the Cyclophosphamide and Control animals at all time-points. We have now perfused, frozen and sliced the brains for days 1, and 14 for all the Doxorubicin animals. Our second group of Doxorubicin for the 56 and 6 month time-points had to be euthanized per our protocol because of rapid and uncontrollable weight loss. We have treated a 3rd round of Doxorubicin animals for the 56 and 6 month time-points (they are not displaying the dramatic weight loss we say in the second day 56 and 6 month groups). We have begun staining for Aims 2 a-d but have only about 1 animal per groups at this point. We anticipate that the staining will be complete for all groups within 3 months with the exception of the third group of animals at the 6 month time-point.

Key Research Accomplishments

- 1) Completed data collection and analysis for the ABR experiments in Aim 2
- 2) Completed majority of data collection and data analysis for the ABR experiments in Aim 1
- 3) Treated, collected, and sliced the majority of the brains for the immunohistochemistry experiments for Aims 1 and 2, begun staining for those Aims, and are continuing to stain until those aims are complete.

Reportable Outcomes

Conferences

Maxwell Hennings, Hawk Cambron, Christy Fessler, Robert Ferguson, and Thane Fremouw. *The auditory brainstem response in mice treated with 5-fluorouracil with antioxidant intervention*. Neuroscience Conference (New Orleans, LA) October 2012.

Maxwell Hennings, Hawk Cambron, Robert Ferguson, and Thane Fremouw. *The effects of single agent doxorubicin, cyclophosphamide, or 5-Fluorouracil treatment on the auditory brainstem response in mice*. Chemo Brain: Mechanisms & Assessments (University of Kentucky, KY) October 2011.

Maxwell Hennings, Hawk Cambron, Robert Ferguson, and Thane Fremouw. *Chemotherapy, Neurotoxicity, and Cognitive Decline: Developing a Mouse Model and Potential Interventions*. Era of Hope Conference (Orlando, FL) August 2011.

Maxwell Hennings, Hawk Cambron, Robert Ferguson, and Thane Fremouw. *Of mice, chemotherapy, and the auditory brainstem response*. Mainely Data (and some theory) (Bates College, ME) May 2011

Maxell Hennings, Hawk Cambron, and Thane Fremouw. *Auditory Brainstem Response in Mice Treated with Doxorubicin or Cyclophosphamide*. Maine Chapter of the Society for Neuroscience Annual Meeting (Bates College, ME) April 2011

We anticipate submitting 3 manuscripts based on our findings from this research.

Personnel Receiving Pay from the Research Effort

The following Faculty, Graduated Students, or Undergraduate Students received pay from the research effort:

Thane Fremouw, Christy Fessler, Maxwell Hennings, Moriah Greer, Teressa Collins, & Thomas Russell

Conclusion

Adjuvant chemotherapy often causes cognitive decline in breast cancer survivors. Although the cognitive deficits are often temporary, it appears that for at least a subset of survivors, the deficits last for years and can have a deleterious impact on survivor quality of life. Recent evidence shows that chemotherapy agents can have long-lasting neurotoxic effects: increase in cell death and decrease in cell division/proliferation in the SVG, the DG, and the CC, as well as delayed myelin degeneration. Which chemotherapy agents or combinations of agents cause CNS damage remains unclear. The results from our auditory brainstem response experiments suggest that 5-Fluorouracil and Cyclophosphamide cause a transient speed of processing deficit, while doxorubicin does not. In addition, our results suggest that co-treatment with antioxidants does not prevent the transient speed of processing deficit. In fact, NAC seemed to cause a prolonged speed of processing deficit. Our immunocytochemistry experiments are ongoing and will help us determine if doxorubicin or cyclophosphamide cause a long lasting decline in neurogenesis and myelination, as does 5-Fluorouracil, and if antidepressants or antioxidants can prevent such damage.